

1    **Activation and induction of antigen-specific T follicular helper cells (T<sub>FH</sub>) play a**  
2    **critical role in LAIV-induced human mucosal anti-influenza antibody response**

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13    *Short title: Activation of antigen-specific T<sub>FH</sub> cells by LAIV*

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26 **ABSTRACT**

27 There is increasing interest recently in developing intranasal vaccines against respiratory tract  
28 infections. Antibody response is critical in vaccine-induced protection and T<sub>FH</sub> is considered  
29 important in mediating antibody response. Most data supporting the role for T<sub>FH</sub> in antibody response  
30 are from animal studies, and direct evidence from humans is limited, apart from T<sub>FH</sub>-like cells in  
31 blood. We studied activation and induction of T<sub>FH</sub> and its role on anti-influenza antibody response by  
32 live-attenuated influenza vaccine(LAIV) in human nasopharynx-associated lymphoid tissue(NALT).  
33 T<sub>FH</sub> activation in adenotonsillar tissues were analysed by flow-cytometry, and anti-  
34 hemagglutinin(HA) antibodies examined following LAIV stimulation of tonsillar mononuclear  
35 cells(MNC). Induction of antigen-specific T<sub>FH</sub> by LAIV was studied by flow-cytometry for induced  
36 T<sub>FH</sub> and CD154 expression. LAIV induced T<sub>FH</sub> proliferation which correlated with anti-HA antibody  
37 production, and T<sub>FH</sub> was shown critical for antibody response. Induction of T<sub>FH</sub> from naïve T cells by  
38 LAIV was shown in newly induced T<sub>FH</sub> expressing BCL6 and CD21, which was followed by the  
39 detection of anti-HA antibodies. Antigen specificity of LAIV-induced T<sub>FH</sub> was demonstrated by the  
40 expression of antigen-specific T cell activation marker CD154 upon challenge by H1N1 virus antigen  
41 or HA. LAIV-induced T<sub>FH</sub> differentiation was inhibited by BCL6, IL21, ICOS and CD40 signalling  
42 blocking respectively, and that diminished anti-HA antibody production. Conclusion: We  
43 demonstrate for the first time the induction of antigen-specific T<sub>FH</sub> by LAIV in human NALT that  
44 provide critical support for anti-influenza antibody response. Promoting antigen-specific T<sub>FH</sub> in  
45 NALT by intranasal vaccines may provide an effective vaccination strategy against respiratory  
46 infections in humans.

47

48 **IMPORTANCE.** Airway infection such as influenza is common in humans. Intranasal vaccination has  
49 been considered a more biologically relevant and effective way of immunization against airway  
50 infection. Vaccine-induced antibody response is crucial for protection against infection. Recent data  
51 from animal studies suggest one type of T cells, named T<sub>FH</sub> is important for the antibody response.  
52 However, data on whether this T<sub>FH</sub>-mediated help for antibody production operates in humans is  
53 limited, due to the lack of access to human immune tissue containing the T<sub>FH</sub>. In this study, we  
54 demonstrated the induction of T<sub>FH</sub> cells by an intranasal influenza vaccine in human immune tissue  
55 that provide critical support for anti-influenza antibody response. Our findings provide direct  
56 evidence that T<sub>FH</sub> cells play a critical role in vaccine-induced immunity in humans, and suggest a  
57 novel strategy to promote such cells by intranasal vaccines against respiratory infections.

58 **Keywords:** T follicular helper cell (T<sub>FH</sub>), LAIV, influenza vaccine, mucosal immunity, antibody  
59 response, nasopharynx-associated lymphoid tissue (NALT)

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## 70 INTRODUCTION

71 Vaccination is one of the most effective preventative measures against pathogenic infection. Despite  
72 its success, there are still many infectious diseases in humans that lack effective vaccines. New  
73 strategies to improve vaccine immunogenicity are constantly being explored. Recent studies suggest  
74 a critical role for T follicular helper cells ( $T_{FH}$ ) in vaccine-induced immunity (1, 2) and promoting  
75  $T_{FH}$  has been considered a promising vaccination strategy. However, most of the current evidence  
76 supporting the importance of  $T_{FH}$  in vaccination comes from animal studies, and direct evidence from  
77 humans is limited, apart from the detection of  $T_{FH}$ -like cells from human peripheral blood samples  
78 which are thought as  $T_{FH}$  equivalent (3, 4). Whether this  $T_{FH}$ -mediated critical help for vaccine-  
79 induced B cell antibody response operates in humans remain largely unsubstantiated. Several recent  
80 studies have reported that the presence of “ $T_{FH}$ -like” cells in peripheral blood following parenteral  
81 influenza vaccination appeared to correlate with an anti-hemagglutinin (HA) antibody response (5,  
82 6).

83  $T_{FH}$  are a subset of CD4+ T cells in secondary lymphoid tissue that provide help to cognate B cells  
84 for high affinity antibody production in germinal centers (GC) and for long-term humoral  
85 immunity(7).  $T_{FH}$  express chemokine receptor CXCR5 and inducible costimulator-ICOS, IL21 and  
86 the transcription factor B-cell lymphoma 6 (BCL6) (8). Considering the importance of  $T_{FH}$  for B cell  
87 antibody response, novel vaccines to induce/activate  $T_{FH}$  cells may be an effective vaccination  
88 strategy for better vaccine efficacy in humans.

89 Influenza virus infects nasopharyngeal mucosa by binding its surface HA to sialic acid receptors on  
90 the host cell (9). Intranasal vaccination has been proposed as an effective way of immunising against  
91 influenza through induction of anti-HA antibody, which relies on the local mucosal immune tissue,  
92 i.e. nasopharynx-associated lymphoid tissue (NALT) as the induction site for immunity. Human  
93 adenoids and tonsils are major components of NALT and are known to be major induction sites for

94 both mucosal and systemic immunity against upper respiratory tract pathogens including influenza  
95 virus (10-13).

96 Live Attenuated Influenza Vaccines (LAIV) are administered as intranasal sprays and comprise of  
97 live-attenuated influenza type A (H1N1 and H3N2), and type B viruses. LAIV has been used in a  
98 number of countries including USA and Canada (FluMist<sup>®</sup>) (14), and in Europe (Fluenz<sup>™</sup>), and been  
99 shown to induce both mucosal and serum antibodies, as well as cellular immune responses (15-17).

100 Although LAIV has been shown to be effective against influenza (18), limited data are available on  
101 the induction of LAIV-induced immunity in humans and on how the anti-HA antibody response is  
102 regulated by T cells. We have studied the activation and induction of T<sub>FH</sub> by LAIV and its role on the  
103 anti-HA antibody response in human NALT tissue, and shown the induction of antigen-specific T<sub>FH</sub>  
104 in NALT is critical in LAIV-induced anti-influenza HA antibody response.

105

## 106 **RESULTS**

### 107 *LAIV activates a proliferative T<sub>FH</sub> response in NALT that provides critical help for anti-HA* 108 *antibody production*

109 Activation of T<sub>FH</sub> in NALT was examined by LAIV stimulation of adenotonsillar MNC for 3 days  
110 followed by enumerating T<sub>FH</sub> numbers using flow cytometry. As shown in Figure 1a+b, LAIV  
111 stimulation elicited a significant increase in T<sub>FH</sub> number (CD4<sup>+</sup>CXCR5<sup>hi</sup>ICOS<sup>hi</sup>) compared to  
112 unstimulated control (p<0.01). The T<sub>FH</sub> response was further assessed by analysis of T cell  
113 proliferation using CFSE cell tracing. As can be seen in Figure 1c+d, stimulation of tonsillar MNC  
114 by LAIV elicited a marked T<sub>FH</sub> proliferative response detected at day 5 of cell culture (p<0.001).  
115 Further analysis also demonstrated a marked increase in the number of germinal center B cells  
116 (CD19<sup>+</sup>CD38<sup>+</sup>IgD<sup>-</sup>) following LAIV stimulation (Fig 1e+f, p<0.01).

117 Anti-influenza antibody production was measured in tonsillar MNC culture supernatant following  
118 LAIV stimulation for 8 days. As expected, LAIV elicited marked anti-HA antibody production (Fig  
119 1g), and T- B cell co-culture experiment demonstrated B cells co-cultured with purified T<sub>FH</sub> elicited  
120 anti-HA antibody production, whereas no antibody production was shown in B cells co-cultured with  
121 non-T<sub>FH</sub> (CXCR5<sup>-</sup>CD4<sup>+</sup>) cells (Fig 1h).

### 122 ***Induction of antigen-specific T<sub>FH</sub> by LAIV that correlates with antibody production***

123 To determine whether LAIV induces T<sub>FH</sub> differentiation from naive CD4<sup>+</sup> T cells in NALT, tonsillar  
124 MNC depleted of CD45RO<sup>+</sup> T cells (resulting in CD45RO<sup>-</sup> MNC) were stimulated for 7 days with  
125 LAIV. The CD45RO<sup>-</sup> MNC contained naive T cells but without CD45RO<sup>+</sup> cells including CXCR5<sup>+</sup>  
126 T<sub>FH</sub>. As shown in Figure 2a+b, LAIV stimulation of CD45RO<sup>-</sup> MNC induced a marked increase in  
127 the number of CD4<sup>+</sup>ICOS<sup>+</sup>CXCR5<sup>+</sup> (T<sub>FH</sub>) cells following 7 days of cell culture. The induced T<sub>FH</sub>  
128 were shown to express the transcription factor BCL6 and cytokine IL21 (Fig 2c+d). The induction of  
129 T<sub>FH</sub> by LAIV was shown in a dose-dependent fashion (Fig 2e, top), which was accompanied by a  
130 dose-dependent increase in anti-HA IgG antibody production in the cell culture supernatant detected  
131 at day 14 (2e, bottom). All the 3 major antibody isotypes including IgG, IgM and IgA anti-HA  
132 antibodies were detected in the culture supernatant at day 14 following LAIV stimulation (Fig 2f).

133 Having shown the induction of T<sub>FH</sub> by LAIV, we next examined the specificity of these induced T<sub>FH</sub>  
134 for influenza antigens. As CD154 is considered a reliable functional marker for antigen-activated T  
135 cells, i.e. a marker for antigen-specific T cells (5, 19-21), CD154 expression in the CD4<sup>+</sup> T cell  
136 subsets was analyzed following either an inactivated sH1N1 virus antigen or recombinant HA  
137 challenge. A representative dot plot demonstrating the activated T<sub>FH</sub> (ICOS<sup>+</sup>CXCR5<sup>+</sup>, top right  
138 quadrant) following the antigen challenge was shown in Fig 3a, and the frequencies of activated T<sub>FH</sub>  
139 (% of CD4<sup>+</sup> T cell) following sH1N1 antigen or HA challenge were shown in Fig 3b. Both antigen

140 stimulations activated a marked increase in the  $T_{FH}$  numbers compared to non-antigen control, and as  
141 expected, the sH1N1 virus antigen challenge elicited a higher increase in  $T_{FH}$  frequency than HA  
142 (3b). Among the activated  $T_{FH}$  cells following sH1N1 challenge, a large proportion (mean 62.2%)  
143 expressed CD154 (3c+d), demonstrating the high frequency of activated influenza antigen-specific  
144 T cells in these  $T_{FH}$ , substantially higher than the other non- $T_{FH}$  CD4<sup>+</sup> cell populations: 0.45% in  
145 ICOS<sup>-</sup>CXCR5<sup>-</sup>, 3.05% in ICOS<sup>-</sup>CXCR5<sup>+</sup>, and 20.6% in ICOS<sup>+</sup>CXCR5<sup>-</sup> populations ( $p<0.001$ ,  
146  $p<0.001$  and  $p<0.01$  respectively) (Fig 3c+d). A similar proportions of CD154<sup>+</sup> CD4<sup>+</sup> T cell  
147 populations including CD154<sup>+</sup>  $T_{FH}$  were shown following the HA antigen challenge (data not shown).

148 ***LAIV-activated induction of  $T_{FH}$  in NALT involves IL21, ICOS, CD40 and BCL6 signalling,***

149 As LAIV induced  $T_{FH}$  cells expressed a high level of IL21 and ICOS, we determined whether the  $T_{FH}$   
150 induction from naïve T cells involved IL21R and ICOS signalling. Co-incubation of naïve T cell-  
151 containing CD45RO<sup>+</sup> MNC with either IL21R or ICOS-Ligand blocking antibody led to a marked  
152 reduction in  $T_{FH}$  cell induction by LAIV respectively (Fig 3e,  $p<0.01$ ). Further, co-incubation with  
153 CD40-ligand blocking antibody or a BCL6 inhibitor also led to a marked reduction in the  $T_{FH}$   
154 induction (3e). Finally, co-incubation with anti-IL21R, ICOS-L and CD40-L antibodies or the BCL6  
155 blocker respectively inhibited the LAIV-induced anti-HA antibody production in CD45RO<sup>+</sup> MNC  
156 (3f,  $p<0.01$ ).

157 ***IL-21 production by LAIV-activated  $T_{FH}$  is critical for anti-HA antibody production***

158 We next examined the cellular source and production of IL21 in tonsillar MNC following LAIV  
159 stimulation, and its effect on  $T_{FH}$  activation and antibody production. Among tonsillar lymphocytes,  
160  $T_{FH}$  were shown as a predominant source of IL21 (4a). Following LAIV stimulation there was an  
161 increase of IL21-producing  $T_{FH}$  in tonsillar MNC (Fig 4b), together with a marked increase in IL21  
162 concentration in the MNC culture supernatant (4c). Further, the increase in IL21 concentration was

163 shown in the co-culture of T<sub>FH</sub> and B cells (4d), but not seen in the co-culture of non-T<sub>FH</sub> with B cells  
164 following LAIV stimulation (4e).

165 IL21 receptor blocking using anti-IL21R antibody abrogated the increase of T<sub>FH</sub> number in tonsillar  
166 MNC elicited by LAIV stimulation (4f), followed by a significant reduction in anti-HA antibody  
167 production in tonsillar MNC (4g).

#### 168 *Activation of T<sub>FH</sub>-like cells in PBMC by LAIV*

169 Recent studies suggest there are T<sub>FH</sub>-like cells in peripheral blood that express CXCR5 and ICOS and  
170 have similar B cell-help functions (5, 22-25). To determine whether LAIV activate T<sub>FH</sub>-like cells and  
171 antibody production in peripheral blood, freshly isolated PBMC were stimulated by LAIV for up to  
172 14 days followed by flow-cytometry and antibody detection. As shown in figure 5a+b, LAIV  
173 stimulation induced an increase of T<sub>FH</sub>-like (CXCR5<sup>+</sup>ICOS<sup>+</sup>) CD4<sup>+</sup> T cells in PBMC (at day 7),  
174 followed by the detection of anti-HA IgG and IgM antibodies in the PBMC culture supernatants (Fig  
175 5c). The activation of influenza antigen-specific T<sub>FH</sub>-like cells by LAIV was demonstrated by the  
176 finding that a major proportion (mean 45.6%) of these cells expressed CD154 following the H1N1  
177 antigen challenge, markedly higher than the other non-T<sub>FH</sub> cell populations (Fig 5d).

#### 178 **DISCUSSION**

179 LAIV is thought to replicate in upper respiratory tract to induce immunity through the local immune  
180 tissue NALT, and it was shown to replicate in nasal epithelial cells(26). As part of the mucosal  
181 immune system in human nasopharynx, adenotonsillar tissue has a surface reticular epithelial cell  
182 layer in which epithelial cells mixed with other cells including a large number of B cells. Many B  
183 cells infiltrating the epithelial layer exhibit memory B cell markers and have great antigen-presenting  
184 potential(27, 28). In our adenotonsillar MNC culture, the predominant cell populations are  
185 lymphocytes of which over 50% are B cells(29). We previously showed a Modified Vaccinia



186 Virus Ankara(MVA) vectored influenza vaccine predominantly infected tonsillar B cells which were  
187 also the major cells presenting vaccine antigens(30). It is likely tonsillar B cells are a major cell  
188 population involved in LAIV replication and antigen presentation to T cells, and this B and T cell  
189 interaction contributes to the vaccine-induced response in NALT. Our recent pilot data showed a  
190 time-dependent increase in HA expression in tonsillar B cells following LAIV stimulation, consistent  
191 with virus replication in tonsillar B cells. Fetal bovine serum(10%) was used in our cell culture, and  
192 we did not find any evidence suggesting blockade of LAIV replication(data not shown).

193 In this study, we have demonstrated the activation and induction of antigen-specific  $T_{FH}$  in human  
194 nasopharynx immune tissue by LAIV, and show  $T_{FH}$  are critical for LAIV-induced B cell anti-HA  
195 antibody response in the immune induction tissue of children and adults.

196 We showed a marked increase in  $T_{FH}$  number in tonsillar MNC following stimulation by LAIV (Fig  
197 1a+b). With CFSE cell tracing, we also demonstrated  $T_{FH}$  proliferation following the stimulation (Fig  
198 1c+d). The increase in  $T_{FH}$  number was accompanied by the production of anti-HA antibodies in  
199 tonsillar MNC (Fig 1g). We further demonstrated in the cell co-culture experiment that purified  $T_{FH}$   
200 from tonsillar MNC helped B cell anti-HA antibody production, whereas non- $T_{FH}$  cells did not (Fig  
201 1h). These results support that  $T_{FH}$  provide critical help for LAIV-induced B cell anti-HA antibody  
202 production in human NALT.

203 Together with the increase in  $T_{FH}$  and antibody production following LAIV stimulation, a marked  
204 increase in GC B cells was also seen in tonsillar MNC (Fig 1e+f). This is consistent with the  
205 assumption that LAIV activates  $T_{FH}$  which support GC B cell proliferation and differentiation for  
206 antibody production. We reported previously that the number of  $T_{FH}$  correlated with that of GC B  
207 cells in NALT (20). These are concordant with previous reports in mouse models that GC B cells  
208 correlated with the appearance of  $T_{FH}$  after influenza virus infection (31) and the magnitude of  $T_{FH}$   
209 response was directly correlated with the GC B cell response (32, 33).

210 We next examined the induction of influenza antigen-specific T<sub>FH</sub> from naïve T cells by LAIV using  
211 T<sub>FH</sub>-depleted CD45RO<sup>-</sup> MNC. 7 days following LAIV stimulation, we have observed a dose-  
212 dependent increase in the number of newly differentiated T<sub>FH</sub> (CXCR5+ICOS+) that co-expressed  
213 BCL6 and IL21, which was followed by the detection of anti-HA antibody at day 14 (Fig 2a-e). Both  
214 BCL6 and IL21 are known to be essential for T<sub>FH</sub> differentiation from naïve T cells in animal studies  
215 (8, 34, 35). Our results here support T<sub>FH</sub> induction in human immune tissue also requires BCL6 and  
216 IL21. Indeed, further experiment with BCL6 blocker and IL21 blocking antibody demonstrated  
217 marked reduction of T<sub>FH</sub> induction from naïve tonsillar T cells, confirming a critical role of BCL6  
218 and IL21 in T<sub>FH</sub> induction. We also showed ICOS signalling blocking inhibited ICOS activation and  
219 T<sub>FH</sub> induction, supporting that ICOS activation is required in T<sub>FH</sub> differentiation. It has been suggested  
220 that CD4<sup>+</sup> T cells utilize ICOS:ICOSL interactions to upregulate IL21 production through which to  
221 contribute to T<sub>FH</sub> induction (35). Our finding that CD40L blocking antibody abrogated T<sub>FH</sub> induction  
222 is in line with the hypothesis that B and T cell cognate interaction through CD40:CD40L signalling  
223 is critical in T<sub>FH</sub> induction.

224 One finding we observed was that CD45RO<sup>+</sup> cell depletion, which removes memory T cells from  
225 tonsillar MNC, markedly reduced anti-HA antibody production analysed at day 8 (for memory  
226 response). The fact that anti-HA IgG level could be readily detected at a high level in whole tonsillar  
227 MNC following vaccine stimulation at day 8 (Fig 1g), whereas in memory T cell-depleted MNC the  
228 antibody production could only be detected at around day 14 at a lower level (Fig 2f) suggests the  
229 presence of influenza-specific memory T/B cells in tonsillar MNC. In this study, although tonsillar  
230 tissues were from non-vaccinated donors, it is likely some of the donors had experienced an influenza  
231 infection previously, and had influenza-specific memory T/B cells. Therefore the presence of the  
232 memory T cells including T<sub>fh</sub> in tonsillar MNC helped the memory B cell response following LAIV  
233 stimulation.

234 Further to the reduction of T<sub>FH</sub> induction following BCL6, IL21, ICOS and CD40L signalling  
235 blocking, we showed that the blockade of these signalling led to a diminished anti-HA antibody  
236 production, supporting the critical involvement of these pathways in T<sub>FH</sub> induction and T<sub>FH</sub>-mediated  
237 B cell antibody production. The induction of influenza antigen-specific T<sub>FH</sub> by LAIV was  
238 demonstrated by the detection of antigen-specific CD4<sup>+</sup> T cell activation marker CD154, which was  
239 expressed in a large proportion of the induced T<sub>FH</sub> following influenza antigen challenge (Fig 3). This  
240 finding is consistent with the report by Bentebibel *et al* demonstrating the increase in influenza  
241 antigen-specific T<sub>FH</sub>-like cells in peripheral blood following an inactivated vaccine immunization in  
242 humans (5).

243 Studies in animal model demonstrated a critical role of IL21 in T<sub>FH</sub> activation and T<sub>FH</sub> were also  
244 shown to be the predominant source of IL21(34, 36). We showed here that stimulation of tonsillar  
245 MNC with LAIV activated a marked increase in IL21-producing T<sub>FH</sub> and in IL21 concentration in  
246 the cell culture supernatant. These results are consistent with the assumption that T<sub>FH</sub> are a major  
247 cellular source of IL21 in human tonsillar lymphocytes, as we found no significant IL21 production  
248 in the absence of T<sub>FH</sub> in the T-B cell co-culture experiment (Fig 4). We also demonstrated that newly  
249 differentiated T<sub>FH</sub> following LAIV stimulation expressed a high level of IL21 (Fig 2). As tonsillar  
250 T<sub>FH</sub> were also known to express IL21R (35), this co-expression of IL21 and IL21R by tonsillar T<sub>FH</sub>  
251 supports the hypothesis that IL21 acts in an autocrine-loop fashion in the vaccine-induced T<sub>FH</sub>  
252 differentiation and function in human NALT. Indeed, we showed that blocking IL21 signalling by an  
253 IL-21R neutralizing antibody inhibited both activation and differentiation of T<sub>FH</sub> induced by LAIV,  
254 and that diminished the anti-HA antibody production. So our results provide direct supporting  
255 evidence that IL21 is crucial in vaccine-induced T<sub>FH</sub> differentiation, and in T<sub>FH</sub>-dependent B cell  
256 antibody production in human immune tissue.

257 Consistent with recent reports that there was an increase in T<sub>FH</sub>-like cells in human peripheral blood  
258 following parenteral influenza vaccination which correlated with the anti-HA antibody response (5,  
259 6), we showed LAIV stimulation of PBMC also induced an increase in CXCR5<sup>+</sup> T<sub>FH</sub>-like cells together  
260 with the production in anti-HA antibodies in the PBMC (Fig 5). The activation of influenza antigen-  
261 specific T<sub>FH</sub> in PBMC by LAIV was demonstrated by the expression of antigen-specific T cell  
262 activation marker CD154 upon antigen challenge. These results support the concept that there are  
263 T<sub>FH</sub>-like cells in peripheral circulation which are functionally similar to T<sub>FH</sub> found in lymphoid tissue  
264 such as NALT, and provide help to B cells for antibody production in an IL21- and ICOS-dependent  
265 manner (22).

266 In conclusion, we demonstrate for the first time the induction of antigen-specific T<sub>FH</sub> in human  
267 immune tissue by an intranasal influenza vaccine, and show its critical role in the anti-influenza HA  
268 antibody response. Our results suggest promoting antigen-specific T<sub>FH</sub> in human NALT by intranasal  
269 vaccines may provide an effective vaccination strategy against respiratory infections in humans.

270

## 271 METHODS

272 ***Patients and samples.*** Patients (age 2–30 years) undergoing adenoidectomy and/or tonsillectomy due  
273 to upper airway obstruction were recruited, and adenotonsillar tissues obtained following operation.  
274 A peripheral blood sample was also obtained before operation. The tonsillar tissues were transported  
275 in HBSS medium (Hank's Balanced salt solution) to the laboratory. Tissue samples exhibiting any  
276 signs of gross inflammation were excluded. Patients with any known immunodeficiency were  
277 excluded from the study. Subjects who received influenza vaccination previously were also excluded  
278 from the study. The Liverpool Paediatric Research Ethics Committee approved the study  
279 [08/H1002/92] and written informed consent was obtained in each case.

280 ***LAIV vaccine and influenza antigens.*** An intranasal LAIV (FluMist, 2009-10) that included  
281 A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains was obtained from  
282 BEI resources (ATCC, Manassas, VA). 0.2ml of LAIV contains approximately  $10^7$  fluorescent focus  
283 units (FFU) of each strain, and we used 2 $\mu$ l/ml ( $\sim 10^5$  FFU/ml) in cell stimulation which was a  
284 predetermined optimal concentration for the activation of anti-HA antibody response following dose  
285 titration experiments. An inactivated seasonal A/Brisbane/59/2007 H1N1 influenza virus (sH1N1)  
286 antigen, which was inactivated by  $\beta$ -propiolactone and partially purified (37) was obtained from the  
287 National Institute for Biological Standards and Control (NIBSC, UK). This inactivated sH1N1  
288 antigen contained 83 $\mu$ g/ml of HA. A purified recombinant HA of sH1N1 (ATCC) was used for HA  
289 antigen stimulation as well as the coating antigen for anti-HA antibody measurement by ELISA. The  
290 recombinant HA contained a C-terminal histidine tag and were produced in High Five insect cells  
291 using a baculovirus expression vector system, purified from cell culture supernatant by immobilized-  
292 metal affinity chromatography (IMAC) and contain a trimerizing (foldon) domain (38).

293 ***Cell culture and stimulation.*** Mononuclear cells (MNC) from adenotonsillar tissues were isolated  
294 using Ficoll density centrifugation (39) (40). In some experiments, tonsillar MNC were depleted of  
295 effector and memory (CD45RO<sup>+</sup>) T cells using CD45RO microbeads and magnetic cell sorting  
296 (Miltenyi) by passing cells through the depletion column twice as described previously (41, 42). The  
297 depletion of CD45RO<sup>+</sup> cells from tonsillar MNC removed T<sub>FH</sub> cells (>98%). Unfractionated whole  
298 MNC or CD45RO<sup>+</sup> cell-depleted MNC were cultured (4x10<sup>6</sup>/ml) in RPMI-1640 medium  
299 supplemented with 10% fetal bovine serum (FBS), streptomycin (50 $\mu$ g/ml) and penicillin (50U/ml)  
300 (Sigma), in the presence the LAIV (2 $\mu$ l/ml unless otherwise stated) for up to 14 days. Cells were  
301 collected at pre-defined time points for analysis of T<sub>FH</sub> cells by flow-cytometry. Cell culture  
302 supernatants were collected for measurement of cytokine and antibody production respectively by  
303 ELISA.

304 ***Flow-cytometry analysis of  $T_{FH}$  cell proliferation and intracellular cytokine expression.*** For  $T_{FH}$   
305 identification, tonsillar MNC were stained with anti-human CD3, CD4, CXCR5 and ICOS antibodies  
306 followed by flow cytometry and  $CD4^{+} CXCR5^{hi} ICOS^{hi}$  cells were identified as  $T_{FH}$  (43, 44). The  
307 tonsillar lymphocytes gated for analysis based on typical FSC/SSC characteristics and singlet  
308 selection has a typical viability >98% viability when examined with propidium iodide staining.  
309 Expression of B-cell lymphoma 6 protein (BCL6), a master transcription factor for  $T_{FH}$  differentiation  
310 (8), in newly induced  $T_{FH}$  cells was analyzed by intracellular staining with anti-human BCL6 antibody  
311 after cell fixation/permeabilization following manufacturer' instructions (eBioscience). Cell  
312 proliferation was examined by Carboxyfluorescein succinimidyl ester (CFSE) staining of tonsillar  
313 MNC (Molecular Probes), followed by cell stimulation for 5 days and by flow cytometry (41, 42).  
314 Briefly, tonsillar MNC were labelled with CFSE (at 37°C, for 8 min) and resuspended in RPMI before  
315 cell stimulation with LAIV (2 $\mu$ l /ml) for 5 days.  $T_{FH}$  cell proliferation was then examined by analysis  
316 of CFSE dilution in  $T_{FH}$  cells ( $CXCR5^{hi} ICOS^{hi}$  cells) by flow cytometry. Intracellular cytokine  
317 expression e.g. IL21 was analysed following a standard intracellular staining procedure including cell  
318 permeabilization as described previously (40). Flow cytometry data analyzed using FlowJo software.  
319 Germinal center (GC) B cell subset was analyzed by flow-cytometry with a combination of CD19,  
320 CD38 and IgD fluorescence-labelled anti-human antibodies and identified as  $CD19^{+}CD38^{hi}IgD^{-}$ .  
321 ***Analysis of antigen-specific  $T_{FH}$  induction.***  $T_{FH}$  differentiation/induction from naïve tonsillar T cells  
322 by LAIV was examined using  $CD45RO^{+}$  cell-depleted MNC (which resulted in  $CD45RO^{-}$  MNC) as  
323 described earlier. The  $CD45RO^{-}$  MNC (with  $T_{FH}$  removed but retained naïve T cell) were stimulated  
324 with LAIV (2 $\mu$ l/ml, otherwise as stated) and cultured for 7 days before flow-cytometric analysis for  
325  $T_{FH}$  cells including CXCR5, ICOS and BCL6 expressions. For the detection of induced influenza  
326 antigen-specific  $T_{FH}$  cells after LAIV stimulation, the cells (at day 7) were washed and incubated for  
327 24 hours in fresh culture medium only, followed by antigen challenge with sH1N1 virus antigen or

328 recombinant HA (1µg/ml) for 6 hours in the presence of brefeldin A. The cells were then stained for  
329 T<sub>FH</sub> including CD4, CXCR5, ICOS, and intracellular CD154 expression after cell  
330 fixation/permeabilization which detects antigen-specific T cells by flow cytometry (19-21).

331 To determine if IL21, ICOS, CD40 and BCL6 signalling pathways are involved in the  
332 activation/induction of T<sub>FH</sub>, neutralizing/blocking antibodies to IL21 receptor, ICOS- and CD40-  
333 ligand (L) or a BCL6 inhibitor were used to co-incubate with tonsillar MNC before LAIV stimulation.  
334 Briefly, recombinant human IL21R-Fc chimera, anti-ICOS-L (R&D systems) and anti-CD40-L  
335 antibodies (InvivoGen) or isotype controls (10µg/ml) or BCL6 inhibitor (79-6, Calbiochem)(50 µM)  
336 were co-incubated with tonsillar MNC or CD45RO<sup>+</sup> MNC for 1 hour prior to stimulation by LAIV.  
337 BCL6 inhibitor 79-6 is a cell-permeable compound that selectively inhibits the transcriptional  
338 repression activity of BCL6. The MNC were then cultured for up to 7-14 days before analysis for T<sub>FH</sub>  
339 and anti-HA antibody production.

340 **Measurement of HA-Specific antibodies.** Production of anti-HA IgG, IgM and IgA antibodies to  
341 sH1N1 virus in cell culture supernatants was measured as previously described (45, 46). In brief,  
342 ELISA plates were coated with recombinant sH1N1 HA overnight. Following blocking, cell culture  
343 supernatants were added and incubated for 2 hours. Alkaline phosphatase-conjugated anti-human  
344 IgG, IgM or IgA antibody was then added and incubated. Following the addition of pNPP substrate,  
345 color development was read at OD405nm at 1 hour and data were analysed using DeltaSoft software.  
346 Intravenous immunoglobulin (IVIG, Intratect ) containing high titers of anti-sH1N1 HA IgG antibody  
347 was used as a reference standard for IgG antibody. Anti-HA IgM and IgA antibody titers were  
348 expressed as OD values (read at 30min) as no reference standard was available.

349 **Cell purification and T<sub>FH</sub>-B cell co-culture.** Tonsillar T<sub>FH</sub> and B cells were purified using magnetic  
350 cell sorting as described previously (43). Briefly, B cells were purified by negative selection using B



cell purification kit (EasySep™, Stemcell) which yielded B cell purity >99%. For T<sub>FH</sub> purification, CD4<sup>+</sup>T cells were first isolated by negative selection, followed by positive selection of CXCR5<sup>high</sup> (T<sub>FH</sub>) using biotin anti-CXCR5 antibody. The amount of anti-CXCR5 antibody was optimised to ensure only CXCR5<sup>high</sup>-expressing cells were selected (purity>95%). CXCR5<sup>-</sup>CD4<sup>+</sup>T (non-T<sub>FH</sub>) cells were isolated by negative selection from CD4<sup>+</sup>T cells using an optimised amount of anti-CXCR5 antibody (purity >99%). Purified B cells were co-cultured (1:1 ratio) with either purified T<sub>FH</sub> or non-T<sub>FH</sub> cells at 5x10<sup>5</sup> cells/ml in the presence of LAIV. The cells were cultured for 10 days and cell culture supernatants were collected for anti-HA antibody analysis.

**Statistical Analysis.** Means and standard errors are used unless indicated otherwise. Differences between two groups were analysed using Student's t test, and paired T test was used for paired samples. Statistical analysis was performed using GraphPad Prism 5 software. P<0.05 was considered statistically significant.

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503

504

505 Figure legends

506 **Figure 1. LAIV induces  $T_{FH}$  proliferation that correlates with GC B cell response and antibody**  
507 **production in NALT.** LAIV stimulation induced an increase in  $T_{FH}$  number (a+b) and  $T_{FH}$   
508 proliferation (c+d) in tonsillar MNC (b & d, n=15, \*\*p<0.01 vs unstimulated medium controls). (a  
509 & c) Representative plots or histogram of  $T_{FH}$  subset (CXCR5<sup>hi</sup>/ICOS<sup>hi</sup>) in CD4<sup>+</sup> T cells following  
510 stimulation (a, day 3), and  $T_{FH}$  proliferation analysed by CFSE (c, day 5, red line: LAIV, grey shaded:  
511 medium control). (e & f) Increase in GC B cell number (CD19<sup>+</sup> CD38<sup>hi</sup> IgD<sup>-</sup>) in tonsillar MNC after  
512 LAIV stimulation (n=13, \*\*P < 0.01 vs control). LAIV-induced anti-HA IgG antibody production in  
513 tonsillar MNC (g, n=20, \*\*p<0.01 vs control, day 8), and LAIV-induced anti-HA IgG production in  
514 B cells co-cultured with  $T_{FH}$  (red bar) or with non- $T_{FH}$  cells (blank bar) (h, n=10, \*\*p<0.01, #p>0.05  
515 vs control). Data in the bar figures are means and SE from a number of different experiments done  
516 with tonsils from different donors.

517

518 **Figure 2. Induction of  $T_{FH}$  from naïve tonsillar T cells and antibody production by LAIV.**  
519 Representative plots (a) and bar graph (b) show the induction of  $T_{FH}$  (CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>) from  
520 CD45RO<sup>-ve</sup> MNC by LAIV compared with medium control (n=10, \*\*p<0.01). (c & d) FACS  
521 histograms of BCL6 (c) and IL21 expression (d) in LAIV-induced  $T_{FH}$  as compared to unstimulated  
522 medium control) (isotype controls: shaded). (e) Dose-dependent induction of  $T_{FH}$  (day 7, top) and  
523 anti-HA IgG antibody production (day 14, bottom) from CD45RO<sup>-ve</sup> MNC following LAIV  
524 stimulation (n=6). (f) LAIV-induced anti-HA IgG, IgM and IgA production in CD45RO<sup>-ve</sup> MNC (day  
525 14, n=10, \*\*p<0.01).

526

527 **Figure 3. Detection of LAIV-induced antigen-specific  $T_{FH}$  and effect of IL21, ICOS, CD40 and**  
528 **BCL6 signalling on  $T_{FH}$  and antibody induction.** CD45RO<sup>-ve</sup> MNC were first stimulated by LAIV  
529 for 7 days followed by influenza antigen challenge with sH1N1 or HA antigen. (a) A representative  
530 plot showing activated  $T_{FH}$  (ICOS<sup>+</sup>CXCR5<sup>+</sup>) following sH1N1 antigen challenge, and (b) showing  
531 the frequencies of activated  $T_{FH}$  (% of CD4<sup>+</sup> T cell) after sH1N1 or HA challenge following prior  
532 LAIV stimulation (\*\*p<0.01, \*\*\*p<0.001 vs LAIV stimulation alone. Medium alone negative

control is also shown). Representative plots (c) and summary frequency (d, n=5) of CD154+ expression in the CD4+ T cell subsets including T<sub>FH</sub> following sH1N1 antigen challenge. (e+f) Effect of neutralizing antibodies to IL21R, ICOS-and CD40-L or BCL6 blocker on T<sub>FH</sub> induction (e, day 7) and antibody production (f, day 14) in CD45RO<sup>ve</sup> MNC following LAIV stimulation (\*\*p<0.01 vs LAIV stimulation or isotype control antibodies).

**Figure 4. IL-21 expression in LAIV-activated T<sub>FH</sub> and its effect on anti-HA antibody production.**

(a) Representative plots showing T<sub>FH</sub> subset and IL21 expression in tonsillar CD4+ T cells following LAIV stimulation (shaded histogram: isotype control). (b) An increase in IL-21-producing T<sub>FH</sub> (% of CD4+ T cells) of tonsillar MNC following LAIV stimulation (n=10, \*\*P < 0.01 vs control). (c-e) IL21 concentrations following stimulation in the culture supernatants of tonsillar MNC (c, n=22), of B cells co-cultured with T<sub>FH</sub> (d, n=10) or with non-T<sub>FH</sub> cells (e, n=10) (\*\*P < 0.01 vs control, NS: not significant). (f+g) IL-21R blocking by adding anti-IL-21R antibody to tonsillar MNC led to a reduction in T<sub>FH</sub> number (f) and in anti-HA IgG, IgM and IgA antibody production (g) (n=8, \*\*p<0.01).

**Figure 5. Activation of T<sub>FH</sub>-like cells in PBMC.** (a) Representative plots shows the increase of T<sub>FH</sub>-like cells (CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>) in PBMC following stimulation for 3 days by LAIV, as compared to medium control. (b) LAIV-induced increase in T<sub>FH</sub>-like cells in PBMC compared with control (n=10, \*\*P < 0.01). (c) Anti-HA IgG and IgM antibody production in PBMC culture supernatant following LAIV stimulation (n=10, \*\*P < 0.01). (d) Frequency of antigen-specific CD154+ T<sub>FH</sub>-like cells (% of CD4+ T cells, red bar) in PBMC following LAIV stimulation and subsequent sH1N1 antigen challenge, compared to other CD4+ T cell sub-populations as indicated (n=4, \*\*p<0.01, \*\*\*p<0.001).













